Biotechnological Approaches to Enhancing Tropical Fruit Quality

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18.1 Introduction

Most fleshy fruits exhibit a high metabolic activity when compared to other plant-derived foods such as seeds. This metabolic activity continues during postharvest storage and makes most fruit highly perishable commodities with short shelf life. It is estimated that around 50% of all fresh fruits and vegetables are lost due to such spoilage, but the exact figure is difficult to determine (FAO, 2006). Fruit quality parameters such as deficiency in flavor and aroma development, reduced shelf life, rapid softening and spoilage, sensitivity to low temperatures, and increased susceptibility to pathogen infection are all a consequence of postharvest deterioration and are major constraints to the availability of fruits. Postharvest problems have been partially solved for many commercial crops (particularly those of temperate climate) by harvesting them at the mature or green stage or by applying various physical and chemical treatments to the ripe fruits and storing them at low temperatures or in controlled atmospheres. However, many fruits, particularly tropical fruits, cannot be handled successfully using these methods. Many tropical fruits harvested at full maturity do not store well, but if harvested at the immature stage, they fail to ripen adequately. In addition, they are very susceptible to low temperatures. Tropical fruits are important in the diet of people in less developed countries, and are increasingly important as exports from many of these countries.

Genetic improvement of tropical fruit crops has a range of objectives, including generation of cultivars with (a) tolerance of biotic and abiotic stresses, with reduced size and altered shape (apical dominance) to increase orchard plant density, lower harvesting and pruning costs, shorten the unproductive period, and improve availability of radiation at the canopy, (b) simultaneous ripening for mechanical harvesting, (c) reduced juvenility period, and (d) higher nutritional value (sugar, oil, vitamins, flavonoids, etc.) to improve the organoleptic qualities and shelf life of fruits.

Conventional breeding of perennial tropical fruit cultivars has been limited by their long juvenile period (up to 20 years), low fertility, high levels of heterozygosity, various levels of ploidy, polyembryony, complex intraspecific incompatibility relationships, and severe inbreeding depression (Gómez Lim and Litz, 2004). Genetic diversity within many tropical fruit crop species is unexplored, and most cultivars are either seedlings from uncontrolled pollinations or dooryard selections. The production of many tropical fruit crops
is based on a rather limited number of cultivars, which are poorly characterized for genetic traits.

Molecular and biotechnological approaches involving genetic transformation, which is the main subject of this chapter, offer an attractive alternative to conventional genetic improvement. In what follows, a description will be made of the different attempts of application of biotechnology for improvement of tropical fruit.

18.2 Fruit ripening

Fruit ripening is a highly complex process, with marked variations in metabolism occurring between different types of fruits. Nevertheless, the process is characterized by a series of coordinated biochemical and physiological changes that lead to the development of (in many cases) a soft, edible fruit (Giovannoni, 2004). Some of these changes include synthesis of secondary metabolites associated with flavor and aroma, synthesis of pigments, degradation of chlorophyll, alterations in organic acids and cell wall metabolism, and a softening of the fruit tissue. At the molecular level, there are a large number of tightly regulated genes involved in specific processes in a highly coordinated manner (Giovannoni, 2004).

In general, fruits are classified as climacteric or nonclimacteric depending on their patterns of respiration and ethylene synthesis during ripening. Climacteric fruits are characterized by an increased respiration rate at an early stage in the ripening process accompanied by autocatalytic ethylene production. Many of the economically important fruit crops are climacteric, and therefore a large amount of research has been devoted to studying the biochemical and molecular pathways operating during the climacteric ripening of fruits. Nonclimacteric fruits, on the other hand, show a different respiratory pattern and display a lack of autocatalytic ethylene synthesis. Research in nonclimacteric fruit has been traditionally lagging behind climacteric fruit, and although there is considerable information, a clear picture of the mechanisms governing the ripening process in this class of fruit is still missing (Adams-Phillips et al., 2004).

Most of the research aimed at modifying ripening has centered on manipulation of fruit firmness (membrane and cell wall properties) and ripening rate (ethylene production or perception) in climacteric fruit. These two aspects are discussed at length in other chapters of this book and will not be discussed here. Instead, the discussion will focus on approaches for improving fruit quality.

18.3 Genetic transformation of tropical crops

Most of tropical fruit crops are perennial trees and, because of the limitations described above, genetic transformation seems to be the only practical solution for improvement of specific horticultural traits (Gómez Lim and Litz, 2004). Genetic transformation provides the means for modifying single horticultural traits without altering the phenotype. This capability is particularly valuable for perennial plants and tree species in which development of new cultivars is hampered by their long generation time. Targeting specific gene traits is predicated on the ability to regenerate elite selections of what are generally trees from cell and tissue cultures. The integrity of the clone would thereby remain unchanged except for the altered trait. However, the difficulty in regenerating many tree species from elite or mature-phase selections is one of the most serious obstacles for applying gene transfer
technologies to these plants (Petri and Burgos, 2005). In addition, very few genotypes of a particular species have been transformed and, in many instances, these genotypes are not commercially important (Petri and Burgos, 2005). With the tools available, it is currently possible to genetically alter practically any gene, as long as a suitable molecular probe is available, and analyze the function during fruit ripening. The ability to apply this technology to various fruits is expanding as the number of fruit-bearing plants that can be transformed and regenerated is also increasing. Currently, the list includes apple, muskmelon, papaya, strawberry, banana, raspberry, mango, avocado, and other tropical and subtropical crops species (Gómez Lim and Litz, 2004).

Evaluating the performance of transgenic fruit tree cultivars requires approximately 12 years or until fruiting and flowering have been observed, depending on the species (Gómez Lim and Litz, 2004). Thus, molecular breeding represents a highly efficient approach for developing improved, perennial fruit cultivars. At present, insertion of foreign genes into plant DNA occurs in a random fashion, which may lead to accidental inactivation of nontarget genes and to variable and unpredictable expression of the transgene itself and even to gene silencing (Kohli et al., 2003). The use of matrix attachment region (MAR) sequences has been proposed to minimize transgene silencing and uniformize transgene expression (Allen et al., 2000). MARs are DNA sequences that bind to the cell’s proteinaceous nuclear matrix to form DNA loop domains. Transgenes flanked with MARs are thought to be able to form their own chromatic domain and thus be insulated from the influences of factors in the chromatin adjacent to its site of insertion (Hall et al., 1991). Because a large majority of plant chromatin is in an inactive conformation at any given time, insulating the transgenes with MARs may reduce the incidence of gene silencing and enhance transgene expression (Lorence and Verpoorte, 2004).

The usual approach is to produce a large number of lines derived from independent transformation events and to select the best genotype among the transformants. A description of the current methods employed to transfer foreign genes to fruit crops is beyond the scope of this chapter, and the interested reader is referred to recently published reviews (Lorence and Verpoorte, 2004; Cotsaftis and Guiderdoni, 2005).

18.4 Metabolic processes related to fruit quality susceptible to manipulation by genetic transformation

In the past, traditional plant breeding centered on improving crop yield. However, consumers are increasingly paying more attention to product quality and composition. As an example, consumers prefer fruits with increased nutritional properties (vitamins, sugars, proteins, minerals, etc.) and ingredients that may help reduce the risk of certain cancer or cardiovascular disorders (e.g., lycopene and antioxidants). These requirements have increased the need for fruits with a longer shelf life and increased quality. In what follows, a discussion of different metabolic processes related to fruit quality that have been or can be genetically manipulated will be made.

18.4.1 Lipid metabolism

Even though they are not a main component of fruits, except avocado, lipids are actively metabolized during ripening, and they can even be employed as indicators of the progress
of the process. There seems to be a correlation between the pattern and timing of ripening in relation to the appearance of lipid peroxidation products, which involves free radical formation, with ethylene treatment significantly inducing their appearance (Meir et al., 1991).

Genetic manipulation of the lipid composition could potentially improve the nutritional value of fruits, the profile of aroma compounds, and the ability of plants to withstand low-temperature stress. At present, two strategies have been used to modify lipid composition in higher plants: (a) alteration of the major fatty acid level by suppressing or overexpressing a specific key enzyme in lipid biosynthesis and (b) synthesis of a fatty acid not found in the host plant. The identification of desaturases, enzymes that introduce a cis-double bond in saturated fatty acids, has led to the production of plants with an increased level of polyunsaturated fatty acids (Arondel et al., 1992) or increased chilling tolerance (Ishizaki-Nishizawa et al., 1996; Khodakovskaya et al., 2006). By suppression of oleate desaturase, the levels of oleic acid (C18:1) in transgenic soybean and of stearate (C18:0) in transgenic canola were increased up to 80 and 30%, respectively (Baldoni and Rugini, 2001). An example of strategy (b) could be seen in canola that does not naturally produce laurate (C12:0), whereas transgenic canola contained laurate by introduction of the proper enzyme (Baldoni and Rugini, 2001).

In avocado, whose outstanding compositional feature is its high fat content, changes in lipids during ripening, including increases in the monoglyceride and free fatty acid fractions, probably result from degradation of triglycerides (Kikuta and Erickson, 1968). Lipid metabolism has been linked with color and flavor development of fruit crops during ripening. Storage lipids may be involved in some manner in the metabolic processes taking place during ripening (Seymour and Tucker, 1993). The majority of lipids found in many fleshy fruits are esters of long-chain fatty acids. An increased fatty acid oxidizing activity has been recorded in some fruits as ripening proceeds (Baqui et al., 1977). The level of total lipids does not normally change during ripening, but the concentration of individual fatty acids (particularly linoleic and oleic acids) may be altered in a particular manner depending on the fruit (Wade and Bishop, 1978; Meir et al., 1991). Products of β-oxidation are used in the synthesis of both carotenoids and terpenoid volatiles (Baker et al., 2006), which are important aroma components of many fruits. Interestingly, mRNA of one enzyme of the β-oxidation pathway, peroxisomal thiolase, has been found to be induced during mango fruit ripening (Bojórquez and Gómez Lim, 1995). This probably reflects an increased β-oxidation pathway activity during ripening whose products are important for aroma production. Recently, acyl CoA oxidase, the key enzyme of β-oxidation, has been identified and also found to be induced during fruit ripening (A. Nila and M.A. Gómez-Lim, unpublished results). Therefore, the role of these enzymes might be to metabolize fatty acids to produce volatiles compounds. This is an area barely explored.

The activity of alcohol: acyl CoA acetyl transferase has been correlated with fruit ripening and the production of aroma volatile compounds in fruits (Shalit et al., 2001). It is possible that the production of those compounds may be increased or modified by genetic engineering.

18.4.2 Cold tolerance

Many fruits are sensitive to low temperatures, particularly tropical products. However, many plants have the ability to increase freezing tolerance in response to low temperature, a
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process known as cold acclimation (Thomashow, 1999). Plants use a wide array of proteins to protect themselves against low temperature and freezing conditions (Shinozaki et al., 2003). There have been many approaches aimed at inducing tolerance to low temperatures, based on traditional breeding as well as on horticultural practices and genetic manipulation. Two examples of the latter (the use of desaturases and the alternate oxidase) are discussed elsewhere in this chapter. Many cold-responsive genes have been identified, and some of them are also induced in response to other types of stress, which suggest that they belong to a family of genes responding to a stress signals in general. However, there also genes specifically involved in the response to low temperatures such as the small CBF gene family encoding transcription factors (Zarka et al., 2003). In Arabidopsis, the transcriptional factors CBF1, CBF2, and CBF3 (also referred to as DREB1b, DREB1c, and DREB1a, respectively) are rapidly induced by low temperature followed by expression of CBF-targeted genes, the CBF regulon, which acts to bring about an increase in freezing tolerance (Sharma et al., 2005). The genes induced by the CBF family contain the CCGAC core sequence also named C-repeat (Baker et al., 1994), which has been found to be essential for the low-temperature responsiveness of additional cold-induced plant genes, including the Arabidopsis gene COR15a (Baker et al., 1994), the Brassica napus gene BN115 (Jiang et al., 1996), and the wheat gene WCS120 (Ou et al., 1998).

Overexpression of CBF3 in Arabidopsis mimics the response of the plant during cold acclimation (Gilmour et al., 2000). The CBF1, 2, and 3 proteins, though highly similar in amino acid sequence, are not identical but they share redundant functional activities (Gilmour et al., 2004). Most of the work on CBF genes has been performed in Arabidopsis, and even though tomato contains three CBF homolog genes, tomato cannot cold acclimatize raising the question whether it has a functional CBF cold response pathway. Only the tomato LeCBF1 gene, however, was found to be cold inducible, and constitutive overexpression of LeCBF1 in transgenic Arabidopsis plants induced the expression of CBF-targeted genes and increased freezing tolerance indicating that LeCBF1 encodes a functional homolog of the Arabidopsis CBF1-3 proteins (Zhang et al., 2004). However, constitutive overexpression of either LeCBF1 or AtCBF3 in transgenic tomato plants did not increase freezing tolerance (Zhang et al., 2004). It is concluded that tomato has a complete CBF cold response pathway, but that the tomato CBF regulon differs from that of Arabidopsis and appears to be considerably smaller and less diverse in function. It remains to be seen whether the other fruit crops contain the CBF regulon and whether it works as in Arabidopsis.

Antifreeze proteins are found in a wide range of overwintering plants where they inhibit the growth and recrystallization of ice that forms in intercellular spaces (Griffith and Yaish, 2004). Unlike antifreeze proteins found in fish and insects, plant antifreeze proteins have multiple, hydrophilic ice-binding domains. Surprisingly, antifreeze proteins from plants are homologous to pathogenesis-related proteins and also provide protection against psychrophilic pathogens (Sharma et al., 2005). Transferring single genes encoding antifreeze proteins to freezing-sensitive plants lowered their freezing temperatures by approximately 1°C (Breton et al., 2000). The identification of these freezing tolerance-associated proteins and the elucidation of their cryoprotective functions will have important applications in several fields (Atici and Nalbantoglu, 2003). Designing new strategies to improve cold tolerance in crop varieties could increase the plant productivity and also expand the area under cultivation.
One example of the above is grapevines. The fruit and wine industries need to maintain and potentially expand production despite increasing constraints in the form of pests, diseases, and temperature stress. However, this must be done without compromising the quality traits of the crop. Using the CBF genes described above, grapevines tolerant to low temperatures were recently generated (Fischer et al., 2004).

18.4.3 Protein metabolism

Research in this area has included the increase of essential amino acid content, the expression of storage protein genes in plant organs other than seeds, and the reduction of the content of allergenic proteins, by genetic manipulation. Work has been done, for example, on the transfer of genes-encoding proteins rich in essential amino acids (i.e., methionine and lysine) from other species. To improve the nutritional quality of soybean, a methionine-rich 2S albumin from the Brazil nut (Betholletia excelsa) has been introduced into transgenic soybeans (Nordlee et al., 1996). However, since the Brazil nut is a known allergenic food, the resultant transgenic soybean turned out to be allergenic as well. For that reason, methionine-rich proteins from sources not containing known allergens have been employed in similar experiments. A maize gene encoding the protein zein increased methionine content by over 80% in transgenic soybean seeds (Baldoni and Rugini, 2001). An amaranth (Amaranthus hypochondriacus) 11S globulin, one of the most abundant storage proteins (amarantin) of the seed, was transferred into tropical maize (Rascón-Cruz et al., 2004). Total protein and essential amino acids of the best expressing maize lines increased 32 and 8–44%, respectively, compared to nontransformed plants. To increase the content of lysine in potato, Sevenier et al. (2002) employed two approaches: Introduction of a feedback-insensitive gene from Escherichia coli (lysCM4) involved in biosynthesis of the aspartate family of amino acids resulted in a sixfold increase of the lysine content, whereas introduction of a mutated form of the key plant enzyme of lysine biosynthesis (dihydrodipicolinate synthase) led to a 15-fold increase in lysine. Allergenicity could also be reduced by genetic transformation. The 14–16-kDa allergenic proteins from rice have been reduced by using the antisense technology (Tada et al., 1996), and the same strategies could be applied in fruit crops.

18.4.4 Flavor and carbohydrate metabolism

Considering the importance of flavor in fruits, it is surprising that no major advances have been made to identify flavor components and enzymes responsible for their biosynthesis in fruits. This might be a reflection of the complexity of this trait. Since many fruits contain high quantities of carbohydrates, particularly sucrose, genetic manipulation of the sucrose-metabolizing enzymes might provide a way to alter sugar content and, in turn, sweetness of fruit.

There are a large number of reports on the manipulation of different enzymes involved with carbohydrate metabolism. Transgenic plants have been produced containing invertase in the sense and antisense orientations (Roitsch and Gonzalez, 2004), antisense granule-bound starch synthase (Liu et al., 2003), antisense phosphorylase (Duwenig et al., 1997), sense and antisense ADP-glucose pyrophosphorylase (Rober et al., 1996; Weber et al., 2000), sense and antisense sucrose synthase (Fernie et al., 2002), antisense uridine diphosphate-glucose pyrophosphorylase (Zrenner et al., 1993), and sense and antisense
sucrose phosphate synthase (Worrell et al., 1991; Strand et al., 2000). All the resultant plants showed an altered carbohydrate content, and considering the potent effect, this process may have on plant metabolism, in general, the use of fruit-specific promoters to target the transgenes to fruit cells becomes imperative.

Another approach to enhance fruit flavor could be the use of a variety of sweet-tasting proteins, thaumatin, monellin, mabinlin, pentadin, brazzein, curculin, and miraculin (Faus, 2000; Sun et al., 2006). All of these proteins have been isolated from plants that grow in tropical rain forests. They elicit a sweet taste by binding specifically with taste receptors and are approximately 100,000× sweeter than sugar on a molar basis (Van der Wel and Arvidsson, 1978; Faus, 2000). The taste-modifying protein, miraculin, has the unusual property of being able to modify a sour taste into a sweet taste.

Transgenic plants have been generated containing monellin (in tomato) (Peñarrubia et al., 1992) and miraculin (in lettuce) (Sun et al., 2006), inducing sweet tasting phenotypes, and a thaumatin gene has been introduced into potato (Witty and Harvey, 1990), cucumber (Szwacka et al., 2002), pear (Lebedev et al., 2002), tomato (Bartoszewski et al., 2003), and apple (Dolgov et al., 2004). The main features of the transgenic fruits were a sweet taste and a liquorice aftertaste lasting for a few minutes. Interestingly, thaumatin has been shown to be strongly induced in ripening fruits (Clendennen and May, 1997; Tattersall et al., 1997).

There have also been attempts at looking for substitutes for the natural sweetener sucrose, and the low-molecular-weight fructans, polymers of fructose, have been suggested as an adequate replacement. These compounds resemble sucrose in their organoleptic properties, but are indigestible by humans. In addition, they cannot be used as a carbon source by caries-causing bacteria. To obtain high fructan plants, the gene encoding 1-sucrose—sucrose fructosyl transferase from *Helianthus tuberosus* was introduced into sugar beet (Sevenier et al., 2002). The transgenic plants showed a dramatic change in the nature of the accumulated sugar, 90% of the sucrose being converted into low-molecular-weight fructan.

### 18.4.5 Plant architecture

Modification of plant architecture by genetic manipulation is now a reality. This result has been achieved by overexpression of phytochromes. The phytochromes are a family of photoreceptors that function as photoreversible pigments in plants. By overexpressing phytochrome A, dramatic and beneficial effects have been obtained in transgenic rice plants such as significant reduction in plant height (which would facilitate harvesting), an increased number of panicles per plant (resulting in a 6–21% higher yield), early flowering, up to 30% more chlorophyll in the leaves (increased photosynthesis in the field), and finally the transgenic lines accumulated significantly more biomass per plant (Robson et al., 1996; Robson and Smith, 1997; Garg et al., 2005). Clearly, this is an area that ought to be explored further.

### 18.4.6 Flower formation

Flower development is an unpredictable and irregular process in many commercial crops. Applications of several chemicals are required to stimulate and coordinate the formation of flowers in several crops. Molecular genetic studies have shown that at least three classes of homeotic genes control the determination of floral meristems and organ identity in
higher plants (Krizek and Fletcher, 2005). Transgenic plants overexpressing genes such as LEAFY or CONSTANS have been generated (Putterill et al., 2004). These genes are sufficient to determine floral fate in lateral shoot meristems with the consequence that flower development is induced precociously.

As mentioned above, most tree species have a long juvenile period of at least 5 years, and the time to evaluate trees can be up to 20 years. This has hampered the development of new, improved cultivars by traditional plant breeding and poses a challenge for the generation of improved varieties when using plant tissue cultures techniques. Transgenic citrus plants overexpressing the genes LEAFY or APETALA1, which promote flower initiation in Arabidopsis, have been produced (Peña et al., 2001). Both types of transgenic citrus produced fertile flowers and fruits as early as the first year, and a shortening of their juvenile period was detectable. Furthermore, expression of APETALA1 was as efficient as LEAFY in the initiation of flowers, and did not produce any severe developmental abnormality. Both types of transgenic trees flowered in consecutive years, and their flowering response was under environmental control. In addition, zygotic and nucellar-derived transgenic seedlings had a very short juvenile phase and flowered in their first spring, demonstrating the stability and inheritance of this trait. These results have opened up new avenues for research in genetic improvement of fruit trees.

18.4.7 Color and pigment metabolism

The external color of fruit is an important factor in consumer preference. The principal pigments in many fruit are carotenoids and anthocyanins, which are synthesized via the terpenoid and phenylpropanoid pathways, respectively. Pigment synthesis manipulation by genetic means represents an interesting choice to modify the color of a fruit to make it more attractive to the consumer. In some cases, the goal would be to increase the color of the transgenic product. Color is particularly important in fruits to be employed for jams, marmalades, pastes, and even wine.

There have been a number of attempts to increase the content of carotenoids in fruit by genetic manipulation, not so much to alter the normal color of plant organs but for other reasons (discussed below), and as expected, many of these attempts have resulted in altered coloration of plant organs. However, there have been some examples of genetic manipulation of carotenoids to alter the color of plant organs. Phytoene synthase, an enzyme induced during fruit ripening, catalyzes the dimerization of two molecules of geranylgeranyl pyrophosphate to form phytoene, the first C40 carotene in the carotenoid synthesis pathway (Römer and Fraser, 2005). Expression of phytoene synthase in antisense in tomatoes produced pale yellow flowers and fruits that ripened to a yellow color (Bird et al., 1991). Lycopene could not be detected in those fruits, although other ripening processes such as polygalacturonase accumulation were unaffected. The carotenoid biosynthesis pathway has also been modified in tobacco plants using the CrtO gene from the alga Haematococcus pluvialis, encoding the β-carotene ketolase (Mann et al., 2000). Transgenic plants accumulated ketocarotenoids that changed the color of the nectary from yellow to red. The authors speculate that plant transformation with this gene may be used in the future to change the color of fruit.

Anthocyanins are flavonoid derivatives which are major secondary plant products well known for the blue, red, and purple coloration they provide to flowers, fruits, and leaves.
Flavonoids are derived from phenylalanine and acetyl CoA in a highly branched pathway leading to flavonols, flavanones, isoflavonoids, and anthocyanins (Forkmann and Martens, 2001). It is known that this complex pathway is regulated at the level of transcription of structural genes (Forkmann and Martens, 2001). In general, genetic manipulation of intermediate enzymes of the flavonoid pathway may change the final balance of these colored compounds and eventually the color of a given plant organ. For example, when a chalcone reductase gene from *Medicago sativa* was introduced in *Petunia*, the flavonoid biosynthesis pathway was redirected since neither chalcone reductase activity nor the product of the reaction, which was further transformed into a colored compound, is naturally present in *Petunia*, and the plant produced yellow flowers (Davies et al., 1998). Similar results have been obtained using different enzymes in other plants (Holton, 1995; Markham, 1996; Su and Hsu, 2003). This illustrates the complex equilibrium of the complete pathway and the difficulty of predictable effects after plant transformation with heterologous genes.

### 18.4.8 Parthenocarpy

The absence of seeds in fruits is a valuable trait, not only from the consumer standpoint, but because it may allow control of fruit development even under adverse environmental conditions for pollination and may be used in fruit crops to standardize and increase fruit size (Gorguet et al., 2005). Horticultural methods for inducing parthenocarpy include spraying of growth regulators, induction of genetic mutations, or modification of ploidy level (Bukovac and Nakagawa, 1967). The parthenocarpy trait is often polygenic and therefore more difficult to deal with in-breeding programs (Gorguet et al., 2005). Parthenocarpy development, in some fruits at least, may be triggered by a deregulation of the hormonal balance in some specific tissues, in particular, between auxins and gibberellins (Fos et al., 2000). An increased level of these hormones in the ovary can substitute for pollination and trigger fruit development. This has been convincingly demonstrated by genetic engineering when the *iaaM* gene, coding for the enzyme tryptophan monooxygenase, was introduced in tomato, tobacco, eggplant, strawberry, and raspberry resulting in parthenocarpic fruits (Rotino et al., 1997; Acciarri et al., 2002; Mezzetti et al., 2004). The *iaaM* gene converts tryptophan to indole acetamide, a precursor of indole acetic acid, and was driven by a placental ovule-specific promoter (DefH9). The expression of chimeric DefH9-*iaaM* starts during early flower development, and the construct mimics the hormonal effects of pollination and embryo development by increasing the content and/or the activity of auxin in the ovule.

Parthenocarpic fruits can also result by mutation of the *pistillata* gene (Yao et al., 2001) or by downregulation of the *sepallata* gene (Ampomah-Dwamena et al., 2002). Expression of the *Agrobacterium rhizogenes* rolB gene in the ovary can also induce parthenocarpy (Carmi et al., 2003). This is an equivalent approach to that of increasing the content of auxins in the ovary as rolB codes for a putative auxin receptor, which makes the plant more sensitive to auxins (Maurel et al., 1994). Finally, high-temperature stress can also result in a seedless phenotype (Young et al., 2004).

### 18.4.9 Nutritional value enhancement

Plants are the staple food for the vast majority of the world’s population, but it is known that they may be deficient in essential nutrients. For that reason, there have been attempts
at increasing the content of various nutrients (vitamins, essential amino acids, flavonoids, lycopene, etc.) by genetic manipulation (Sevenier et al., 2002).

Vitamins are essential factors in the diet, and they must be obtained from the diet. In addition, some vitamins are used as functional additives in food products. The edible part of rice grains, the endosperm, lacks vitamin A, and a diet based mostly on rice consumption may eventually cause vitamin A deficiency (Tucker, 2003). An outstanding achievement has been the introduction of genes into rice that enabled the biosynthesis in the endosperm of \( \beta \)-carotene, the precursor of vitamin A (Ye et al., 2000). The grain of the transgenic rice had a yellow golden color and by itself contained sufficient \( \beta \)-carotene for human vitamin A requirements. The authors of this work have waived all intellectual property rights for exploitation of these technologies in the developing world, and are actively involved in assisting the International Rice Research Institute to breed stable and agronomically successful lines for use in vitamin A-deficient areas. Similar experiments have been performed successfully in rapeseed, where introduction of a phytoene synthase gene also increased the level of vitamin A precursor (Kishore and Shewmaker, 1999) and in tomato, where introduction of a bacterial phytoene desaturase increased the \( \beta \)-carotene content in fruits up to twofold (Römer et al., 2000).

Another lipid-soluble vitamin with an antioxidant role is vitamin E (\( \alpha \)-tocopherol). Daily intake of this vitamin in excess of a recommended minimum is associated with decreased incidence of several diseases. Plant oils are the main source of dietary vitamin E, and they generally have a high content of the vitamin E precursor \( \gamma \)-tocopherol. Overexpression of \( \gamma \)-tocopherol methyl transferase greatly increased the seed level of \( \alpha \)-tocopherol in Arabidopsis (Shintani and DellaPenna, 1998) and corn (Rocheford et al., 2002). Apart from these examples, transgenic plants containing elevated levels of vitamin C have also been produced (Herbers, 2003). These experiments have resulted in functional food with enhanced health benefits, but there are now many laboratories in the public and private sectors looking to achieve vitamin levels high enough in transgenic plants to merit extraction from the plant (Herbers, 2003). Attempts to increase the content of carotenoids in fruit by genetic manipulation are common (Romer and Fraser, 2005; Long et al., 2006), and the reason is because they possess potent antioxidative, photoprotectant, and anticancer properties (Fraser and Bramley, 2004; Hix et al., 2004).

Flavonoids are another group of secondary metabolites whose inclusion in the human diet, in particular the flavonol group (e.g., quercetin and kaempferol), may give protection against cancer and cardiovascular diseases (Chen et al., 1990; Hou, 2003; Ren et al., 2003). The biosynthetic pathway leading to the synthesis of these compounds has been known for a long time, and consequently, the design of strategies to increase the content of these compounds has been possible. For example, transformation of tomato with a gene from Petunia, encoding a chalcone isomerase or with the maize transcription factor genes LC and C1, has resulted in fruits with an increased content of flavonoids (Muir et al., 2001; Bovy et al., 2002). Interestingly, by suppression of an endogenous photomorphogenesis regulatory gene, DET1, by RNA interference technology both carotenoid and flavonoid contents were increased significantly, whereas other parameters of fruit quality were largely unchanged (Davuluri et al., 2005).

When vegetables are the major components in the diet, there is a certain risk of iron deficiency. Although some plants are rich in iron, availability is limited by the oxalic acid and phytate-like substances present in the plant, which may complex this element. Oral
administration of ferritin, a protein used by plants and animals to store iron, can treat anemia in rats. Consequently, soybean gene encoding ferritin, under the control of a seed-specific promoter, has been introduced into rice (Goto et al., 1999). Transgenic rice plants accumulated ferritin in the endosperm and up to threefold levels of iron in comparison to normal seeds. Interestingly, plants overexpressing ferritin appeared to be tolerant to oxidative damage and pathogens (Deak et al., 1999).

Lipids are also important components of the human diet. There is an increased preference for plant-derived oils to the animal fats because of health concerns. Plant oils are mostly used for human consumption as margarines, oils, and food ingredients. Triacylglycerols are the most important components of plant seed oils. Properties such as melting point, color, flavor, mouthfeel, spreadability, stability, and effects on human health are determined by the fatty acid composition of the triacylglycerols.

Nowadays there is a trend toward a reduction of saturated fatty acids in the diet and an increase in unsaturated fatty acids. It has been known for many years that intake of monounsaturated fatty acids is associated with a lowered incidence of coronary artery disease (Keys et al., 1986). Therefore, the unsaturation of fatty acids and the increase of unsaturated fatty acids have been targets for modification by genetic engineering studies. The content of unsaturated fatty acids could be increased in soybean, maize, and canola and potentially in other crops by manipulating the expression of desaturase genes (Kinney et al., 2002). There is now considerable evidence of the importance of n-3 long-chain polyunsaturated fatty acids in human health (Gill and Valivety, 1997). They are normally found in fish oils but plants can be genetically engineered to synthesize these important fatty acids as a sustainable alternative source (Napier and Sayanova, 2005).

18.4.10 Molecular farming

The production of plant-derived biopharmaceuticals is sometimes referred to as molecular farming. The word biopharmaceutical is applied to a naturally occurring or modified polypeptide, protein, DNA, or RNA product that is to be used for therapeutic, prophylactic, or in vivo diagnostic use in humans or animals. The main categories of biopharmaceutical products are proteins, antigens, therapeutic monoclonal antibodies, and polyclonal antibodies. The first report on the production of biopharmaceuticals in plants was published in 1992 (Mason et al., 1992); since then proof of concept has been well established and over 100 products have been expressed in plants, several clinical trials performed, and three plant-based biopharmaceuticals are already in the market (Streatfield et al., 2003; Woodard et al., 2003; Howard, 2004; Dus Santos and Wigdorovitz, 2005). Several cereals, and in particular maize, have been the system of choice for expression of antigenic proteins since the proteins can be expressed at high levels in the kernel and stored for prolonged periods without excessive deterioration (Streatfield et al., 2003). Plants are natural bioreactors, and potentially a cheap source of recombinant products (Fischer et al., 2004). However, one possible inconvenience of using plants as bioreactors for biopharmaceuticals is post-translational modifications introduced by the plant. It is known that plants can glycosylate heterologous proteins and attach a variety of carbohydrates, including some not present in animal cells (Faye et al., 2005). These extra carbohydrates can alter the properties of heterologous proteins. For that reason, a detailed analysis of the plant-based product is
imperative. Fruits are also ideal choices for production of biopharmaceuticals since this can be achieved in a totally contained atmosphere such as a greenhouse.

18.4.11 The alternate oxidase

The alternate oxidase is an enzyme involved in the cyanide-resistant respiratory pathway, and it transfers electrons from the ubiquinone pool to oxygen without energy conservation. The enzyme can use reductants (electron donors) that are produced in excess and cannot be used efficiently by the cytochrome pathway, preventing the formation of reactive oxygen species from an overreduced ubiquinone pool, and thus may be involved in acclimation to oxidative stresses (Umbach et al., 2005) and to low temperatures (Fiorani et al., 2005). In addition, the alternate oxidase may act as an important mitochondrial “survival protein” against programmed cell death (Robson and Vanlerberghe, 2002). It has also been studied in thermogenic species, and its activity correlated with heat production, necessary to volatilize foul-smelling compounds to attract insect pollinators. There is a significant participation of this pathway in the climacteric of many fruit. A cDNA coding for the mango alternate oxidase has been identified, and by northern blot analysis the message was detected in unripe fruit and shown to increase substantially in ripe fruit (Cruz Hernandez and Gómez Lim, 1995). These results showed, for the first time, the participation of this enzyme in fruit ripening at the molecular level and were subsequently confirmed by an independent group (Considine et al., 2001). The temperature in ripe mango pulp is up to 10°C higher than in unripe pulp, and this has been attributed to the activity of the alternate oxidase (Kumar et al., 1990). This extra heat might also serve to volatilize aroma-giving compounds. Unfortunately, no additional studies have been performed in other ripening fruit.

18.4.12 Genetic stability

Considering the time and effort invested in transferring a gene in long-lived perennials such as fruit trees, it is essential that stable patterns of gene expression are maintained for long periods of time. Although fruit trees are normally vegetatively propagated, the transgene should also be expressed in the progeny. There have been several studies to address this issue both with marker genes (Vain et al., 2002; James et al., 2004) or with genes conferring novel agronomic traits, such as rolABC from A. rhizogenes in transgenic kiwi plants of staminate GTH and pistillate Hayward cultivars (Baldoni and Rugini, 2001). After 12 years, the staminate rolABC plants maintained the same morphology and the offspring (transgenic staminate X normal pistillate) was transgenic in 50% of plants. The cherry rootstock Colt, containing Rit-DNA, which seems to modify the scion vigour, showed stability after 4 years in the field (Baldoni and Rugini, 2001). R. Scorza and coworkers have performed extensive analyses on transgenic Prunus domestica carrying the plum pox virus coat protein (PPV-CP), uidA and nptII genes. Gene expression has been stable in the greenhouse for over 5 years and the progeny, produced from hybridization of transgenic plants carrying plum pox virus coat protein, expressed the transgenes (Ravelonandro et al., 1997). These results seemed predictable in the light of a study in Arabidopsis carried out to search for transcriptome changes associated with expression of transgenes regulated by constitutive promoters (El Ouakfaoui and Miki, 2005). Insertion and expression of the marker genes, uidA and nptII, did not induce changes to the expression patterns of the approximately 24,000 genes that
were screened under optimal growth conditions and under physiological stress imposed by low temperatures (El Ouakfaoui and Miki, 2005). This study showed that the transgenic and nontransgenic plants were equivalent in their global patterns of transcription, and it may contribute to the principle of substantial equivalence, which is used as a first step in the biosafety evaluation of transgenic crops.

Apparently, stability of transgenes in the genome of transformed plants depends on their correct physical integration into the host genome as well as on flanking target DNA sequences. The exact site of transgene insertion into a plant host genome cannot, at present, be controlled and is poorly understood. A detailed analysis of transgene integration in 19 independently derived transgenic barley lines was carried out by fluorescence in situ hybridization (Salvo-Garrido et al., 2004). The pattern of transgene integration appeared to be nonrandom, and there was evidence of clustering of independent transgene insertion events within the barley genome. The data from the transgene flanking regions indicated that transgene insertions were preferentially located in gene-rich areas of the genome.

In another study with different transgenic lines of aspen, inverse PCR analysis revealed an additional truncated T-DNA copy of 1,050 nucleotides adjacent to the left border of the complete copy in one of the lines (Kumar and Fladung, 2001). Sequencing of this truncated T-DNA revealed that it represented an inverted copy of part of the right half of the original construct, which would allow the inverted repeat to pair with right border sequences of the complete copy. This would explain the frequently observed reversion resulting in transgene loss due to intrachromosomal base-pairing leading to double-stranded loops of single-stranded DNA during mitotic cell divisions (Kumar and Fladung, 2001).

18.5 Future perspectives

The improvement of fruit crops has depended on various technologies that have had varying degrees of success. Conventional breeding has been very successful with herbaceous species, but improvement of perennial fruit crops by traditional means has been limited. Biotechnologies that could increase the efficiency of fruit crop improvement, in particular tropical crops, are, therefore, essential to generate improved cultivars with novel traits. For example, genetic mapping could provide breeders with the tools to make rapid progress in crop improvement. Functional genomics and proteomics could provide insights into genetic regulation of plant function and novel means for isolating genes for manipulation in transgenic plants. Older biotechnologies, including somatic hybridization, in vitro mutation induction, and selection, have rarely been applied to tropical fruit species for crop improvement. There have been predictions that biotechnology will play a significant role in the twenty-first century (Cantor, 2000).

Even though transgenic plants with improved agronomic traits have already been produced in several fruit species, efforts have mainly focused on resistance to biotic stress and fruit ripening, while less work has been done on, for instance, altering growth rate or providing cold stress resistance. It is likely that the focus for development in the coming years will be on multiple gene introductions to increase output traits such as increased nutritional value, vitamin content, or improved flavor components. Obstacles still exist for some species in fundamental methodology, including gene transfer, genetic selection, and efficient protocols for regeneration. However, it seems possible to overcome these limitations following the recent contribution of a double regeneration system, which allows one
to obtain and maintain morphogenesis in calli for a long time in fruit species such as apple, cherry, and olive (Baldoni and Rugini, 2001).

Work with tropical crops lags far behind that with herbaceous species. Genetic transformation of perennial fruit crops has generally depended on embryogenic systems, and therefore regenerants of the woody species. Many species must pass through a period of juvenility before they can be properly evaluated. Two alternatives have been utilized to overcome this limitation: (1) invigorating plant material through grafting of mature buds onto juvenile stock plants (Cervera et al., 1998); (2) constitutive expression of either the LEAFY or APETALA 1 genes from Arabidopsis thaliana to shorten the juvenile phase and promote precocious flowering (Peña et al., 2001). Both of these innovations could stimulate more transformation attempts with perennial species.

It would be advisable to use new selectable markers instead of the traditional ones and reduce the percentage of loss, which can be as high as 40% in many fruit crops such as apple, pear, banana, citrus, and grape (Gómez Lim and Litz, 2004). It would even be better to get rid of marker genes altogether and to employ one of the several methods available to generate marker-free transgenic plants (Ebinuma et al., 1997; Daniell et al., 2001; Huang et al., 2004; Wang et al., 2005). Clearly, these approaches would help improve public acceptance and perception of transgenic plants.

The major hindrances that have stymied genetic transformation studies with tropical/subtropical fruit, however, concern lack of regeneration protocols for elite (mature phase) selections and the relative absence of molecular studies. The latter reflects the state of the science in many developing countries where tropical fruit crops are grown on a large scale and the relative severity of production and postharvest problems of the crop. Biotechnology studies involving fruit crops everywhere are generally underfunded, and national and international agencies should perhaps consider more support for research with these plants.

References


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